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Original communication

Black and green tea – How to make a perfect crime

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ABSTRACT

The antioxidant properties of the black and green tea are well known. The latent bloodstains are detectable by luminol. The bloodstains also can be cover up by drinks and foods containing the antioxidants; thus their presence can cause a decrease of the luminol light emission (false-negative results). The aim of this study was to quantify the light emission decrease of the chemiluminescent mixture prepared according to Weber (containing NaOH) and the chemiluminescent mixture of pH 7.4 (for the determination of the total antioxidant capacity) for the open air-dried sample. The black and green teas and white wine were used as the antioxidant's samples (high and low total antioxidant capacity). The significant decrease of the luminol chemiluminescent emission caused by the presence of the black and green teas (and comparable for both of them) was observed in comparison with the presence of white wine.

1. Introduction

1.1. Antioxidants

Body cells and tissues are continuously threatened by the damage caused by the reactive oxygen species – ROS (e.g. free radicals), which are produced during normal oxygen metabolism or are induced by exogenous damage.[1] To protect themselves from ROS, living organisms have developed several effective mechanisms. The antioxidant-defense mechanisms of the body include enzymes (such as superoxide dismutase and catalase) but also nonenzymatic counterparts (such as glutathione, ascorbic acid, and α-tocopherol).[1]

The roles of fruit, vegetables and red wine in disease prevention have been attributed, in part, to the antioxidant properties of their constituent polyphenols, vitamins E and C, and the carotenoids. In fact, tea catechins are the most powerful antioxidants among the known plant phenols.[2,3] In some lab tests, EGCG (epigallocatechin 3-gallate) is 20 times more active than vitamin C, 30 times more than vitamin E.[4] Commercially grown teas are hybrids of two distinct ecotypes: the Assam-type (var. *assamica*) and the China-type (var. *sinensis*).[5] The first apical leaves are picked from the evergreen shrub and can be processed by different methods. Green tea is promptly dried with or without a fixation step to inactivate enzymes. Black tea is the result of the oxidation of leaf polyphenols through a multi-stage enzymatic process.

Also flavonoids may have an additive effect to the endogenous scavenging compounds. The best-described property of almost every group of flavonoids is their capacity to act as antioxidants. Fermentation of red wine on the grape seeds and skins allows more extensive extraction of phenolic components such as flavanols, flavonols, and proanthocyanidins than in the case of white wines, for which pomace contact is generally kept to a minimum.[6] The total antioxidant capacity (TAC) of red and white wine usually differs 10 times.[7]

The chemiluminescent antioxidant capacity determination is based on the measurement of the length of inhibition time of luminol dependent chemiluminescent light emission, assuming that it is directly proportional to the total antioxidant capacity. As a standard is often used Trolox (water-soluble equivalent of vitamin E) and the assay is called the Trolox Equivalent Antioxidant Capacity (TEAC).[8,9]

1.2. Bloodstains detection

In case of the visible bloodstains the presumptive colour test (e.g. eosin, Hemastix) is usually used. The bloodstains which are not visible by the naked eyes are detectable by the luminol based methods. The light emission of luminol as an evidence of bloodstains is largely a complex process based on the hydrogen peroxide decomposition catalyzed by haemoglobin and is dependent on several circumstances including pH, temperature and compounds that can interact with luminol or catalyst.[10]

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The hydrogen peroxide decomposition is predominantly the first step of the luminol chemiluminescent reaction. The products of this decomposition – ROS – are able to induce the chemiluminescence of luminol. The kind and amount of this reactive oxygen species are dependent on the type of the catalyst. Three basic types of catalyst for the hydrogen peroxide decomposition are known: enzyme (e.g. horse-radish peroxidase), HClO, and metal ion (e.g. Fe, Cu, Co).

Haemoglobin is the oxygen-carrying molecule found in the erythrocytes of all vertebrates and some invertebrates and is responsible for the red colour of blood. Mammalian haemoglobin is a tetrameric hemoprotein composed of four globins each enclosing a prosthetic heme group, consisting of a protoporphyrin IX–Fe^{2+} coordination complex. As these ferric heme derivatives show the same catalytic properties and capability of participating in two-electron redox cycles as a group of enzymes called peroxidases widely distributed especially in vegetables, their activity is termed a pseudo-peroxidase or peroxidase-like.[11]

Luminol can be used to detect the presence of minor, unnoticed or hidden bloodstains diluted down to a level of $1{:}10^6$ (1 μl of blood in 1 L of solution).[12,13] Two oldest and best known formulations of the luminol mixture for the bloodstain detection (the first one described by Grodsky et al.[10] and the second one described by Weber[14]) continue to be the most extensively used by forensic experienced person. Bluestar® Forensic[15] is a formula that is luminol-based and eliminates the numerous inconveniences (in 2000).

Mostly, the problematic contaminants – for the bloodstains detection – are those that enhance luminol light emission or induce a generation of light even if blood – as a catalyst – is not present (false-positive results).[16] They are e.g. some biological materials containing peroxidase or some households containing chlorine. Especially because of an effort to clean the bloodstains, they were included in many studies. But the bloodstains can also be cover up by drinks and foods – the natural source of the antioxidants. The aim of this work is to determine the conditions under which the false-negative results of the bloodstains detection can be caused by the presence of the antioxidants. The source of them can be the black and green teas because of their high total antioxidant capacity (TAC) and also because of the colour of their infusions. This colour can be confused with the colour of the diluted bloodstains (in the range of the dilution from $1{:}10^3$ to $1{:}10^4$) especially in case when somebody is trying to mask the dried, almost latent bloodstain, by the spillage of the black or green teas.

The initial step of the TEAC determination is identical to the bloodstain detection – the hydrogen peroxide decomposition and the production of the reactive oxygen species. It was found out that the concentrations of luminol and hydrogen peroxide for the TEAC determination and the bloodstain detection described by Weber are identical (the differences are catalyst – horse radish peroxidase *vs*. haemoglobin – and pH – PBS *vs.* NaOH).

2. Materials and methods

2.1. Instrumentation

Fluoroskan Ascent FL – Microplate Fluorescence and Luminescence Reade (Thermo-Labsystems, Finland) – the luminescent kinetic mode (integration time 20 μs), temperature 25 °C.

2.2. Chemicals and reagents

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) sodium salt, H_2O_2, NaOH, NaCl, Na_2HPO_4, KH_2PO_4 were Sigma–Aldrich (Germany) compounds. (PBS – NaCl, Na_2HPO_4, KH_2PO_4, pH 7.4).

2.3. Tea infusions

The stock solution of tea was prepared from 2 g of dry leaves (samples were obtained from common tea rooms: black tea – Earl Grey, green tea – Japan Hojicha) and 100 ml of boiling distilled water. Each sample was mixed for 3 min and then filtered.[17] The samples were stored in the fridge.

2.4. White wine

The white wine was obtained from the common supermarket. The white wine was chosen as a compound with the low TAC. Generally, the TAC of the black or green tea is comparable to the TAC of the red wine and the TAC of the white wine is approximately 10 fold lower then the TAC of the red wine.[7]

2.5. Chemiluminescent mixtures

Chemiluminescent mixture (luminol solution) was prepared according to Weber[14] or by dissolving luminol sodium salt in PBS buffer (Phosphate Buffered Saline – NaCl, Na_2HPO_4, KH_2PO_4, pH 7.4) and the addition of hydrogen peroxide (luminol 0.004 M + hydrogen peroxide 0.176 M).

2.6. Chemiluminescent measurement

The arrangement of the chemiluminescent measurement was identical to the previous paper[17] – blood was used as a catalyst, the antioxidant sample was added and the chemiluminescent reaction was initiated by the injection of the chemiluminescent mixture. The blood was diluted by PBS in the range from $1{:}10^3$ to $5{:}10^9$ (the final dilution in the microplate well). PBS was chosen for this dilution because it is a part of TEAC determination. 10 μl of the blood solution and 50 μl of the antioxidant sample were placed into the well and left on the open air for 24 h to dry. The antioxidant's samples were twice diluted only (it was supposed that the common beverage was spilt out). The chemiluminescent mixture (mixture containing NaOH or mixture containing PBS buffer) was injected into the well to trigger the chemiluminescence. The chemiluminescent light intensity was an average value of three measurements. 100% light emission intensity is the light emission intensity of the sample without the antioxidant.

The catechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate, which occur in black and green teas, are supposed to produce hydrogen peroxide.[18] The measurement by Fluoroskan Ascent FL is not sensitive enough to show the change of the chemiluminescent light emission caused by the additional hydrogen peroxide produced by the teas. The chemiluminescent light intensity in the presence of the black and green teas together with the chemiluminescent mixtures was lower than 1 RLU (RLU – relative light unit).

The chemiluminescent light intensity showed in the figures is an average value of three measurements (the range is from 0 to 5000 RLU, up to the saturation of the measure channel in case of 6000 RLU; it is not possible to show the error bars in the 3-D graphs created by Excel).

The aim was to find out how can the solutions containing antioxidant cover (mask) the latent bloodstain in dependence on their antioxidant capacity – (the TAC of the teas is about 6 fold higher than the TAC of the white wine).[19]

3. Results and discussion

In the forensic bloodstains detection (latent bloodstain), the visual examination by luminol is usually used as a presumptive test. In

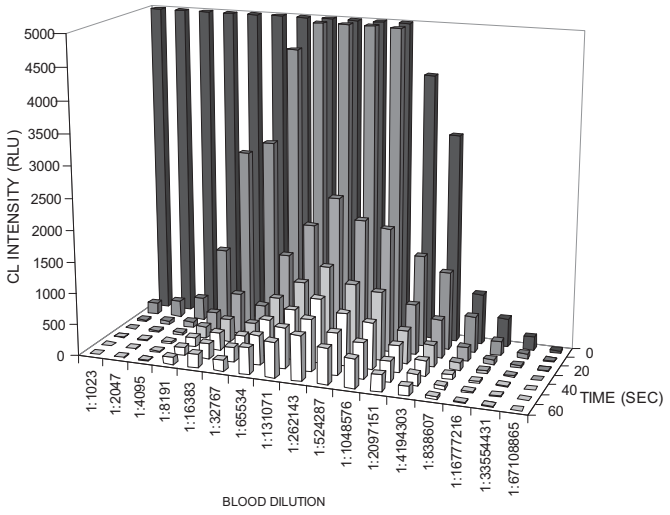

Fig. 1. Dependence of the luminol chemiluminescent intensity (RLU) on the blood dilution. The chemiluminescent mixture was prepared according to Weber; the measurements were done immediately after injection of the chemiluminescent mixture and after following 10, 20, 30, 40, 50 and 60 s.

case of the blood dilution more than 1:5000, the bloodstain is colourless and is detectable by using of Fluoroskan Ascent FL. Nevertheless, the colour of the stain can be caused for example by the tea infusion. The aim of this study was to explore how can the infusions containing antioxidant cover (mask) the latent bloodstain in dependence on their antioxidant capacity and to quantify the luminol chemiluminescent intensity decrease (percentage) measured by using of Fluoroskan Ascent FL in the antioxidant presence.

Figs. 1 and 2 demonstrate the changes of the luminol chemiluminescent intensity in dependence on the blood dilution immediately after injection of the chemiluminescent mixture and after 10, 20, 30, 40, 50 and 60 s.

It was found out, that the blood dilutions of the potentially latent bloodstains correspond to the blood dilutions detectable by Fluoroskan Ascent FL (if the undiluted or less diluted samples were used, the signal was too high and the measure channel was saturated, see Fig. 1 – immediately after the injection and also a few

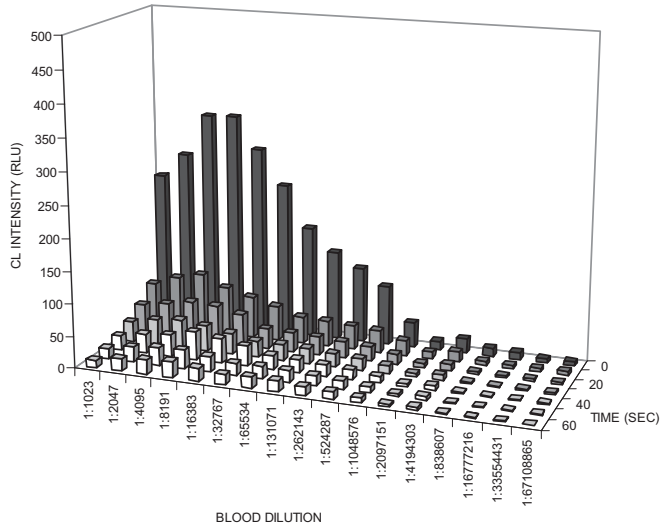

Fig. 2. Dependence of the luminol chemiluminescent intensity (RLU) on the blood dilution. The chemiluminescent mixture was prepared in PBS buffer pH of 7.4; the measurements were done immediately after injection of the chemiluminescent mixture and after following 10, 20, 30, 40, 50 and 60 s.

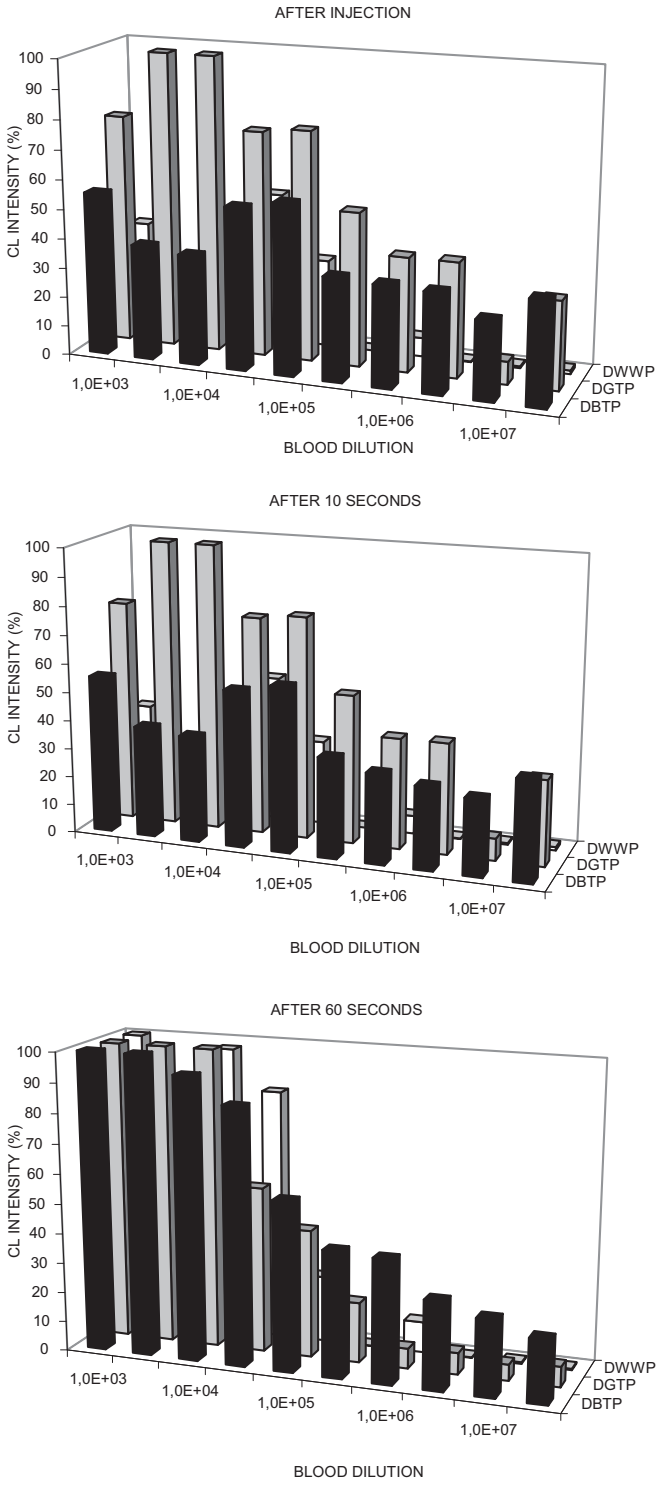

Fig. 3. The quantification of the luminol chemiluminescent intensity decrease in the presence of black tea (BTP), green tea (GTP) and white wine (WWP) (DBTP, DGTP, DWWP – open air-dried samples). The chemiluminescent mixture was prepared in PBS of pH 7.4.

measurements 10 s after the injection). The chemiluminescent intensity of the mixture prepared according to Weber is at least 10 fold higher than the chemiluminescent intensity of the mixture prepared in PBS buffer of pH 7.4.

The following figures (Figs. 3 and 4) demonstrate the intensity changes (percentage, not RLU) of the luminol mixture catalyzed by

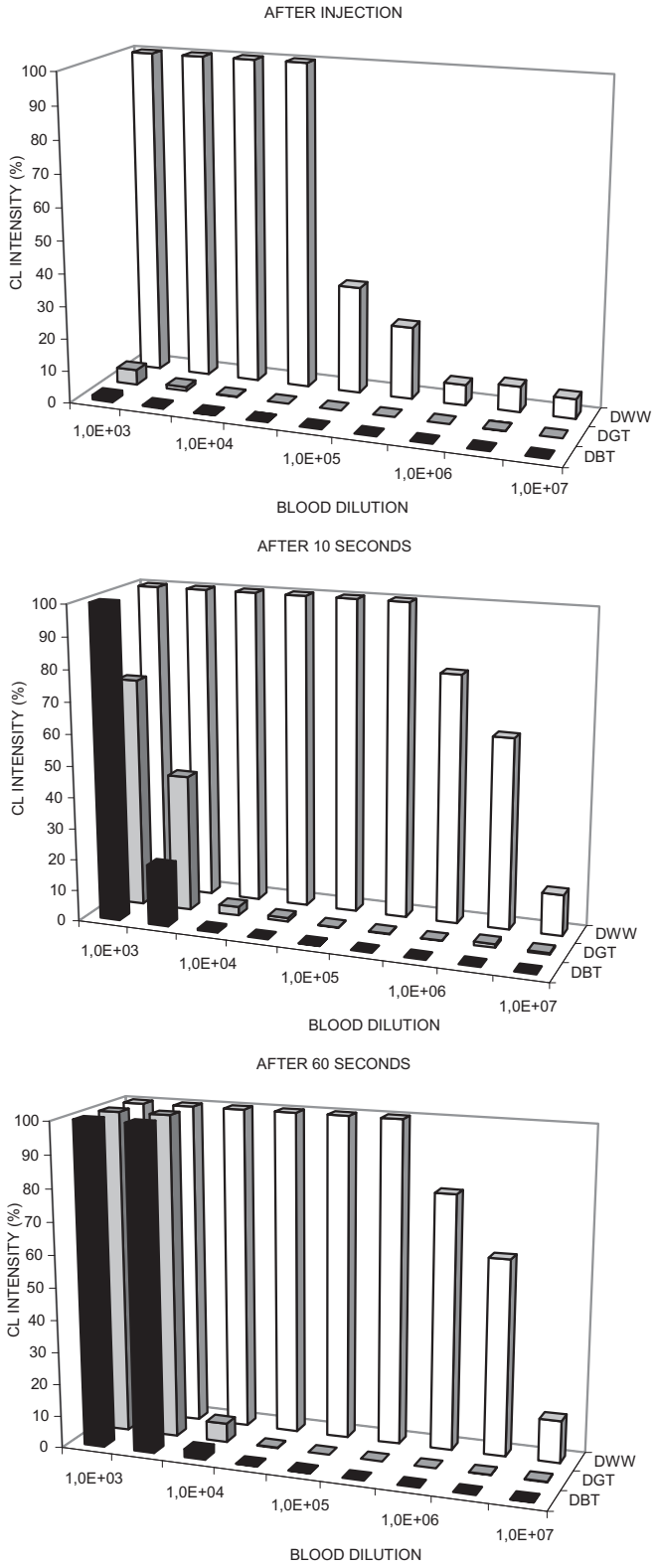

Fig. 4. The quantification of the luminol chemiluminescent intensity decrease in the presence of black tea (BT), green tea (GT) and white wine (WW) (DBT, DGT, DWW – open air-dried samples). The chemiluminescent mixture was prepared according to Weber.

the presence of blood in dependence on the presence of the antioxidant. The black and green teas and white wine were used as the antioxidant samples. The tea infusions, which can cause a stain of the colour similar to the diluted bloodstain, are marked as BT – black tea and GT – green tea; the colourless antioxidant – white wine is marked as WW. There is the comparison of chemiluminescent mixture prepared in PBS buffer of pH 7.4 (see Fig. 3; there is letter P in the antioxidant abbreviation, BTP – black tea sample + chemiluminescent mixture in PBS buffer of pH 7.4) and according to Weber (see Fig. 4). Letter D in antioxidant abbreviation (e.g. DBT) means black tea open-air dried sample.

The samples of the black and green tea can mask the bloodstain and are able to minimize (but not absolutely quench) the chemiluminescent light emission during the bloodstain detection at least during the first minute after spraying. The decrease and the restoring of the light emission are similar to the total antioxidant capacity determination. In case of the black and green tea their abilities to cause false-negative bloodstain detection are comparable and dependent on the blood dilution.

The presence of the black and green teas decreased the chemiluminescent light intensity of the chemiluminescent mixture prepared in PBS buffer of pH 7.4 almost for all blood dilution only in case when the measurement was done 10 s after the injection of the chemiluminescent mixture. The ability of all antioxidant samples to cause the light intensity decreased which can be interchangeable as the false negative results was proved for the blood dilution more than 1: 5.10^5. The efficiency to decrease or to delay the luminol chemiluminescent emission was evidently higher for air-dried samples in the presence of the black and green tea in comparison with the white wine after the injection of the chemiluminescent mixture prepared according to Weber. The comparison of the strong sources of the antioxidants (black and green teas) and weak source of the antioxidants (white wine) has confirmed that the strong antioxidants (especially when the stain colour can be interchangeable) can give false-negative results of the bloodstain detection.

4. Conclusions

The latent bloodstains (dilution more than $1:10^3$) are detectable by the luminol mixture according to Weber and also in PBS solution pH 7.4 by using of Fluoroskan Ascent FL. The light intensity of chemiluminescent mixture prepared in PBS buffer of pH 7.4 is about 10 fold lower then the light intensity of the chemiluminescent mixture prepared according to Weber. But it still allows the use of the luminometr in case when the sample (evidence) is infused by PBS. The antioxidants are present in many drinks and foods; thus they can be the contaminants of the crime scene and the bloodstains can be cover up by them; in case of the dilution more than $1:10^3$ the colour of the stain could be caused for example by undiluted tea infusion. The decrease or delay of the chemiluminescent intensity maximum immediately after injection (or during 60 s after injection) of the chemiluminescent mixture prepared according to Weber in the presence of the antioxidants was demonstrated in all cases. It was found, that all described conditions (pH, blood dilution, antioxidant sample) have influenced the chemiluminescent intensity and it is necessary to take them into account during the bloodstain detection.

Ethical approval

None.

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Conflict of interest

I wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

I confirm that there are no other persons who satisfied the criteria for authorship but are not listed.

I confirm that I have followed the regulations of our institutions concerning intellectual property.

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